Effects of Mold Exposure on Immune Cells

The Honors Program
Senior Capstone Project
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Mold Exposure's Effect on Human Macrophages
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ABSTRACT

The relationship between exposure to mold spores and human disease is only beginning to be understood. While evidence exists of strong correlations between moldy environments and allergic and infectious diseases, the relationship between exposure to specific species and human immune responses to them is not fully understood. This paper seeks to clarify this relationship by analyzing the effects of exposing murine immune cells to volatile organic compounds (VOCs) produced by different mold species. Mold species studied include *Stachybotrys alternans*; tests performed include cell viability studies and immunoassays. Results have implications for further research and treatment of mold-related diseases.

INTRODUCTION

In the five years since hurricane Katrina struck the gulf coast, the health effects of indoor mold has been the subject of much media attention. Stories abound about homeowners who returned to their homes months after the floods receded to find them completely infested with thick coats of visible, malodorous mold colonies – from walls to furniture to carpeting. Disturbingly, this increase in media attention paid to mold problems corresponds with the publication of a recent World Health Organization study that correlates increased risk of respiratory symptoms and infections with damp and moldy environments (WHO, 2009).

"Molds" consist of a variety of fungal species that are common in both indoor and outdoor environments. They are distinguished from other fungal species in their morphology; unlike yeasts, mold species are multicellular and contain distinctive filamentous structures called hyphae. Generally speaking, molds are non-photosynthetic, requiring a nutrient-rich environment for growth, and can reproduce both sexually and asexually. Molds reproduce via sporulation: under the proper conditions, certain hyphal cells produce spores – small, durable particles that contain the mold's genetic information and equipment capable of forming new colonies. Spores are released into the mold's environment and disperse throughout it; under proper conditions they germinate and form new colonies on nutrient-rich substrates (Campbell & Reece, 2005). Such substrates can include cellulose, starch, and lignin, which in indoor environments are found in drywall, fabric, food, and many other household items.

The human immune system consists of a plethora of mechanisms that defend the body from harmful pathogens. These mechanisms can be broken down into two categories: components of the innate immune system, which recognize common microbial characteristics and therefore nonspecifically defend against these pathogens, and those of the adaptive immune system, which recognize microbial components called antigens. Antigens are specific to each pathogen, and therefore adaptive mechanisms are specific to one microbial species; additionally, the adaptive system exhibits characteristics of memory, as subsequent exposures to the same pathogen result in increasingly effective immune responses to it. The adaptive immune memory is responsible for allergies and hypersensitivity diseases; allergic symptoms arise in response to exposure to typically harmless antigens and are essentially an unnecessary

immune response to them. The exact mechanisms of many types of allergies are not fully understood. Typically, immune responses involve cell to cell communication via cytokines – chemicals secreted by immune cells that illicit some type of response in other cells. Chemokines are chemo-attractant cytokines – cytokines that cause immune cells to migrate in specific ways in response to their secretion (Abbas & Lichtman, 2001).

Several different human illnesses are caused by exposure to molds. Generally speaking, these illnesses can be categorized based on the type of immune response elicited by such exposure. Hypersensitivity diseases (such as allergies) involve an immune response to typically harmless microbes, while infectious diseases involve immune responses to actively colonizing pathogens. Additionally, certain organizations, such as the Occupational Health and Safety Administration, classify some molds as "irritants" – substances that invoke a temporary immune response that decreases as exposure decreases (Bush et al., 2006). More specifically, toxins and volatile organic compounds (VOCs) produced by certain fungal species, particularly those associated with damp indoor environments, have been implicated in the condition known as "sick building syndrome" – a long list of symptoms commonly exhibited by occupants and/or workers in some buildings, often those that have been exposed to water via flooding, fire (and thereby sprinkler systems), or some other mechanism.

The recent increase in prevalence in atopic hypersensitivity diseases such as asthma and allergies is a subject of concern for physicians, public health officials, and society in general. Increases in asthma and other atopic diseases have been studied in rapidly industrializing nations, such as former soviet states, with results positively correlating increases in hypersensitivity with conditions of rapid industrialization. "Westernization" of lifestyles in such countries has been implicated as one of many underlying causes of hypersensitivity increases; specifically, factors such as "better house insulation and tighter windows with a subsequent increase in dampness and moulds" may contribute to hypersensitivity in those growing up during the transition to industrialization in East Germany and other nations (Heinrich et al., 2002, p. 1040).

Hypersensitivity diseases associated with mold exposure include atopic conditions such as asthma, allergic bronchopulmonary aspergillosis (ABPA), allergic fungal sinusitis, and

hypersensitivity pneumonitis (HP) (Bush et al., 2006). Chronic rhinusitis (CRS) has been hypothesized to be due to hypersensitivity to certain fungal species, but existing evidence is lacking and antifungal treatments for CRS have shown inconclusive results (Bush, 2005). ABPA typically affects those with asthma or cystic fibrosis and is characterized by a localized hypersensitivity to certain mold species (usually *Aspergillus fumigatus*, but other species have been implicated) that occurs in the lungs and is associated with anatomical changes that make breathing difficult and may cause other complications (Greenberger, 2002). Similarly, allergic fungal sinusitis involves fluid accumulation in the sinuses that foster the abnormal growth of fungi, particularly *A. fumigatus,* and entails significant hypersensitivity to the species present (Luong, 2004). HP is an allergic disease where some sort of allergen (which can be from a diverse set of sources, including mold) is inhaled and the immune system of host is hyposensitized to it. Sensitization and disease generally require exposure to high doses of the allergen, prolonged exposure, or both, and is therefore in most cases occupational. HP is rare, and disease is generally reported only in a small set of occupations including farming and bird keeping, although extremely similar disease is reported in workers exposed to inhaled chemicals as well (Bush et al, 2006).

Infectious diseases caused by molds typically involve superficial infections in certain tissues (i.e. thrush, subcutaneous infections, etc.). These diseases are often present in otherwise perfectly healthy individuals and can be easily treated with antifungal agents; only a very small number of aggressive fungal pathogens can be acquired, and their occurrences are rare and generally depend on host factors, especially immunodeficiency (Bush et al., 2006).

Disease can also be caused by toxic byproducts or metabolites of molds (mycotoxins). Generally speaking, molds that produce mycotoxins are considered toxic. Trichothecene mycotoxins are produced by many taxonomically unrelated mold genera including *Fusarium* and *Stachybotrys* and have been implicated in many health conditions. Many of these trichothecenes are toxic to eukaryotic cells due to their chemical structure; specifically, animal cells exposed to trichothecenes experience inhibition of protein, DNA, and RNA synthesis, impaired mitochondrial function, and disruptive effects on membranes (Rocha et al., 2005). Trichothecenes act as noncompetitive inhibitors of the ribosomal peptidyl

transferase – an enzyme critical for protein synthesis in eukaryotes (Thompson and Wannemacher, 1986). Consequently, as peptidyl transferase inhibitors trichothecenes can also trigger a ribotoxic stress response that activates mitogen-activated protein kinases (MAPKs), which regulate apoptosis under stress (Shifrin & Anderson, 1999). With specific regard to immune responses, trichothecenes trigger inflammatory responses in leukocytes (Kankkunen et al., 2009). Certain trichothecenes have also been shown to induce apoptosis in natural killer (NK) cells, which indicates some cancer-promoting activity as well (Paananen et al., 2000).

Volatile organic compounds (VOCs) are organic compounds that can vaporize under normal conditions into the atmosphere, and are produced by several black mold species. Although literature on trichothecenes is relatively abundant, research on the effects of VOCs produced by molds is less prevalent. Many VOCs produced by molds are known toxins, and several previously ambiguous species have recently been shown to induce signs of neurotoxicity in invertebrates (Bennett, 2010).

This paper seeks to clarify the relationship between mold and human immune responses to it. Because there is both lacking and conflicting evidence regarding the precise effects of mold exposure to the health of individuals and populations, more research is necessary to elucidate such relationships. Molecular immunological analysis of different mold type's effects on human immune cells can result in valuable information regarding these relationships, and combined with other information can yield improvements in overall understanding of diseases caused by molds and their appropriate treatments.

In this particular study, mold spore samples from different locations around Bryant University were collected, and individual species from these samples were isolated. Murine macrophages were exposed to 1-octen-3-ol, a VOC produced by Stachybotrys and other toxic mold species, and the resulting cellular changes were analyzed using cell Trypan Blue exclusion assay and ELISA.

MATERIALS AND METHODS

Collection of Samples at Bryant
Samples were collected using Sabouraud and potato dextrose agars. Sample plates were left open at each location for 72 hours, then closed and allowed to incubate at room temperature for one week. Colonies of interest were chosen based on morphology – black colored colonies were isolated and sent to Air Quality Environmental Laboratory Services (Seminole, FL) for identification; tape lifts were prepared directly from the plates according to the lab's instructions.

Isolation of Splenic Leukocytes
Splenic Leukocytes were isolated as follows. Spleens from 4 uninfected C57BL/6 mice (Jackson Laboratories) were harvested into 6-well plates containing 3ml of PBS/Serum. The spleens were then transferred to 0.3uM cell strainers and mechanically digested through the strainer into 3mL of PBS/Serum. The cellular mixture was centrifuged and washed two times with PBS/Serum. The red blood cells in the resulting cellular pellet were lysed using NH_4Cl. The cells were then washed and centrifuged again. The total leukocytes isolated were counted and re-suspended for separation.

Cell Sorting
Cells were re-suspended at a concentration of 106/100ul in cold PBS/Serum. A PE-labeled F4/80 biomarker was used to sort for monocytes. Cells were incubated with PE-F4/80 and control IgG antibodies for 30 minutes on ice. Following cellular staining, the cells were washed twice with cold PBS/Serum. The stained cells were then sorted using a FACS/Aria (BD Biosciences). Purified monocytes were identified as being positive for F4/80. A total of $7.6X10^6$ cells were isolated.

Trypan Blue Exclusion Assay
1-octen-3-ol was obtained in solution (98%) from Acros Organics and diluted to three different concentrations using RPMI media. For three different concentrations of octenol, $1.36X10^6$ F4/80+ cells were suspended in 10 µl media; the concentrations of octenol used were 0.01% (1 µl), 0.05%, (5 µl) and 0.10% (10 µl), respectively, plus a 0.0% concentration control. Each octenol concentration was tested at six time points – after cell viability was determined, samples were frozen to preserve for ELISA. Viability was determined using

Trypan Blue dye. The dye was incubated with samples for the last three minutes of incubation time. Samples were observed on a hemocytometer, and cell death percentages as proportions of blue (dead) cells to total cells were recorded.

ELISA
ELISA was performed using a murine MCP-1 duokit from BD Biosciences. A 96- well plate was coated with anti-MCP-1 capture antibody and incubated overnight at 4°C. The plate was washed and blocking reagent was incubated for one hour at 37°C. The plate was washed again, and samples and known standards were added and incubated for two hours at room temperature. After another wash, Strep-Avidin HRP was added and incubated for thirty minutes at room temperature. Substrate solution was added and incubated for fifteen minutes to stop reactions, and the plate was read in a spectrophotometer at 450 nm.

RESULTS

Bryant University Samples are Nontoxic
Mold samples from twelve locations around the Bryant University campus were cultured and four samples of interest were sent for identification to Air Quality Environmental, Inc. The samples of interest were from the Bryant Center, the Unistructure, and townhouse N8. Although some samples appeared black, no toxic black mold species were identified (Figure 1).

The molds identified belong to *Cladosporium* and *Penicillium* genera (Table 1). *Cladosporium* spores are common in indoor and outdoor air and are often implicated in hypersensitivity diseases, while *Penicillium* species are equally widespread – one species is responsible for common bread mold. *Penicillium* mycotoxins have also been associated with Organic Dust Toxic Syndrome (ODTS), an occupational disease with flu-like symptoms that occurs mostly in those who work in building remodeling or mold remediation; for the low-exposure lifestyles of the general public, however, *Penicillium* species are typically harmless. The samples also included abundant to significant amounts of hyphae, which are fungal structures that emerge when spores have germinated and are actively growing (Baker, 2010).

1-octen-3-ol Decreases Viability of Macrophages
Given that no toxic fungal species were identified in samples from the Bryant campus, the potentially toxic VOC 1-octen-3-ol was obtained for further investigation. 1-octen-3-ol (also known as octenol) is chemically classified as an alcohol and is a compound associated with the musty odor of mold. In invertebrate toxicity studies of thirteen VOCs produced by various mold species, octenol displayed the most significant levels of toxic effects, killing 100% of *Drosophila melanogaster* subjects within a week of exposure (Bennett et al., 2010). For this reason, octenol seems to be the most promising source of toxicity to cells exposed to mold, and I chose to expose macrophages to it.

To test the viability of macrophages after exposure to 1-octen-3-ol, Trypan Blue exclusion techniques were used.   Cells were exposed at concentrations of 0.01%, 0.05%, and 0.10% and incubated for 24 hours. Cell viability was tested at 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours. As expected, increasing VOC concentration was correlated with increased cell death, and longer incubation periods resulted in higher cell death percentages (Figure 2, Table 2). Effects of cell viability were observed almost immediately (within one hour) and continued until most cells were dead after approximately 24 hours.

1-octen-3-ol Induces MCP-1 Secretion by Macrophages
To further investigate the effects of 1-octen-3-ol on macrophages, chemokine production was analyzed. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that plays an important role in the recruitment of monocytes/macrophages to sites of injury and infection. In viral models, this recruitment occurs via stimulation of the CCR2 chemokine receptor, and increasing concentrations of MCP-1 secreted by immune cells corresponded with the progression of infection (Hokeness et al., 2005).

Like the viability assays described above, MCP-1 level were tested via ELISA at varying concentrations and exposure times. Results were as predicted and congruent with viability results: increasing MCP-1 production corresponded with increasing VOC concentrations and exposure times (Figure 3, Table 3). As in viability tests, significant effects on MCP-1 production were observed within one hour, and production of the chemokine increased steadily over 24 hours.

DISCUSSION

Understanding the mechanisms of diseases caused by molds and their toxins is critical to the development of appropriate and effective treatment strategies. This study represents one of the first immunological tests of the effects of the VOC 1-octen-3-ol on immune cells.

As expected, increasing concentrations and exposure times of 1-octen-3-ol decreased the viability of murine macrophages. Significant decreases in viability were seen immediately, and continued steadily until the vast majority of cells were dead after 24 hours. These results offer some explanation for the immunosuppressive effects of mold exposure – decreasing macrophage populations certainly can affect the body's ability to fight disease in suppressive ways.

Similarly, increasing MCP-1 production by macrophages over time and based on concentrations of 1-octen-3-ol indicate a significant effect on immune function. Since MCP-1 is involved with the recruitment of macrophages to sites of infection, the overproduction of MCP-1 in response to exposure to the VOC represents a possible last-ditch effort by macrophages to call in peripheral immune cells. This overproduction might also contribute to cell viability results described earlier; cytokines can be toxic to immune cells and tissues in large enough doses, and apoptotic cells activated by the cytokine IFN-γ have been shown to overproduce MCP-1 (Yosuke et al., 2002). Increasing concentrations of MCP-1 may also be involved in the kinds of hypersensitive immune responses observed in cases of mold allergies.

Interestingly, the concentrations of MCP-1 observed in this study were notably similar to those in previously studied viral models. This indicates an immune response similar to one during a pathogenic infection – an important finding in the investigation of the health effects of mold exposure. If more such responses are observed in the future, this would suggest that mold exposure is in fact a major health concern, even for those traditionally thought of as invulnerable. Such advances in knowledge will help shape policies on mold prevention and remediation and could thus lead to better health outcomes worldwide.

APPENDICES

Appendix A – Figures
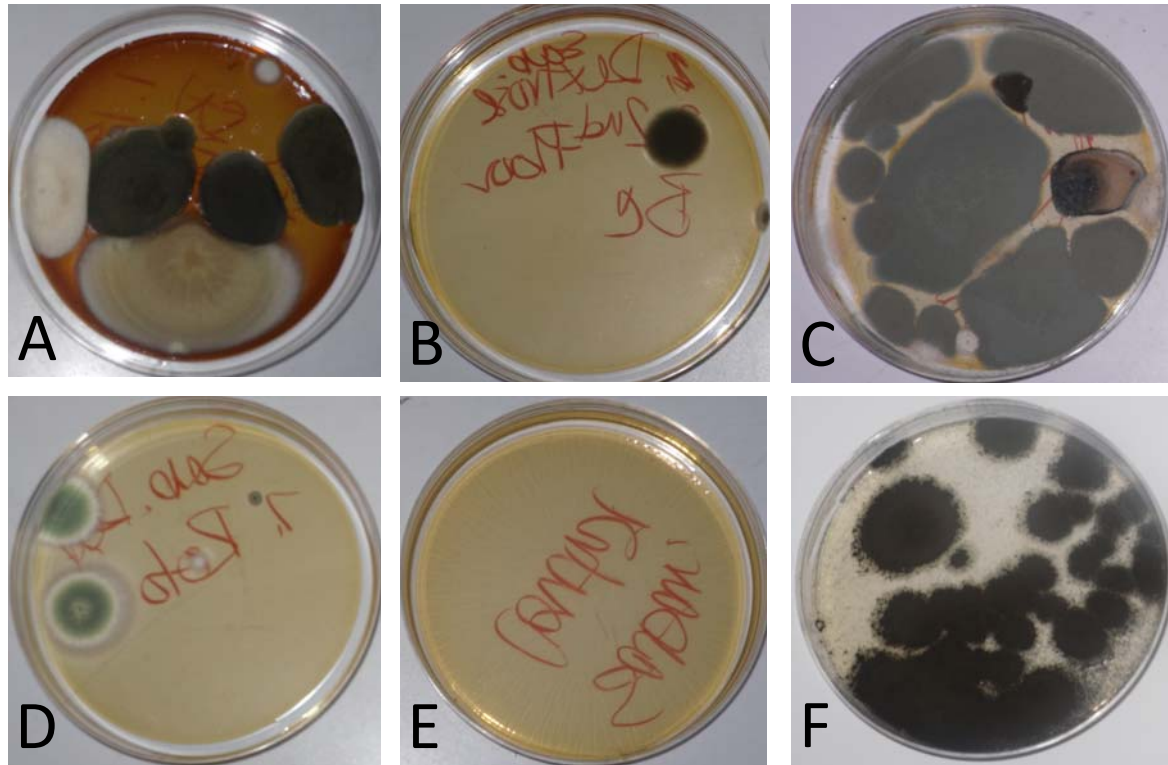
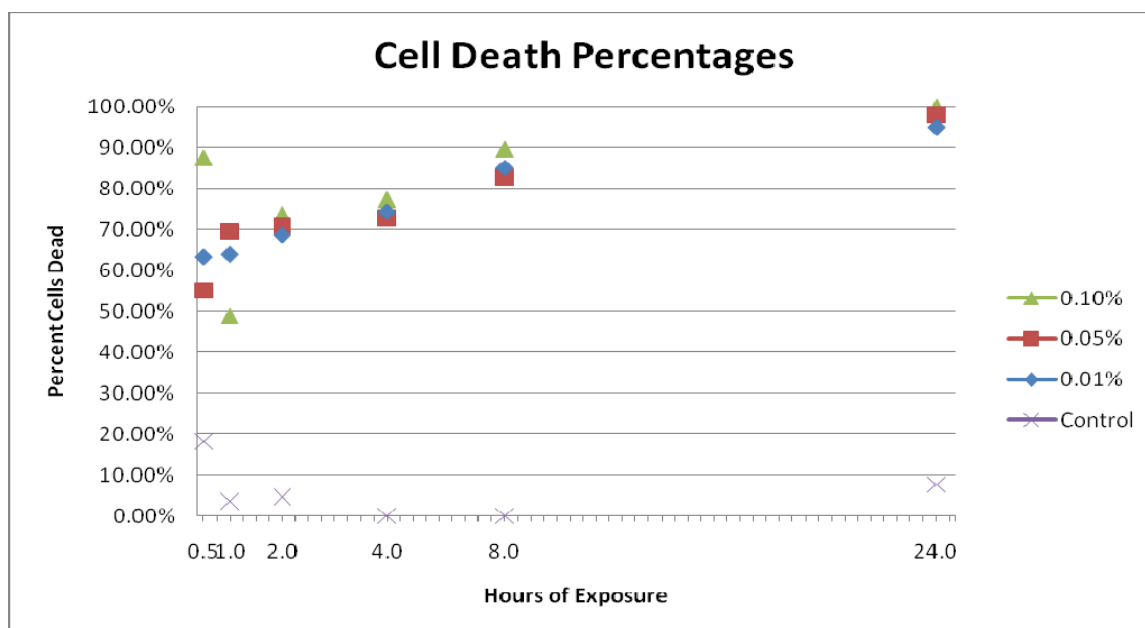**Figure 1**: Sample plates from various locations around Bryant University campus: first floor Bryant Center, second flood Bryant Center, townhouse N8, rotunda (A, B, C, D, respectively); control plate (E); toxic mold *Stachybotrys alternans* (F).

Figure 2: Macrophage viability after exposure to 1-octen-3-ol. Increasing concentrations induced more cell death, as did increasing exposure times.
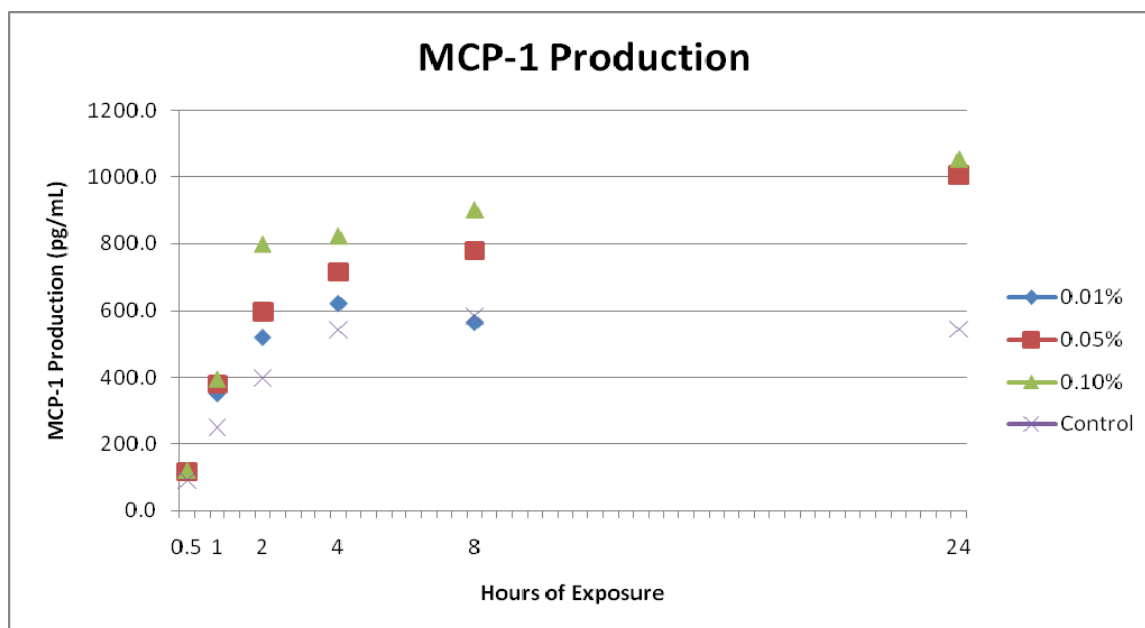
Figure 3: MCP-1 production after exposure to 1-octen-3-ol. Similar to cell death tests, increasing concentrations induced more MCP-1 production, as did increasing exposure times.

Appendix B – Tables

Lab Number	103819	103820	103821	103822
Customer Number	1	2	3	4
Customer ID	Roto	1st Floor BC	2nd Floor BC	N8
Hyphae	Abundant	Abundant	Abundant	Significant
Cladosporium sp.	Sig-Abundant	Sig-Abundant	Abundant	Abundant
Penicillium sp.				Abundant
Total Mold Spores	Sig-Abundant	Sig-Abundant	Abundant	Abundant

Table 1: Molds identified from Bryant University campus; "significant" indicates some limited contamination (2-5 spores per field), while "abundant" indicates too many spores to count.

Hrs. of Exposure:		0.5		1		2		4		8		24	
		Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2
0.01%	Dead	13	7	29	21	44	79	35	19	12	7	11	9
	Total	17	14	55	28	80	96	36	37	13	9	11	10
Avg. % Dead:		63.24%		63.86%		68.65%		74.29%		85.04%		95.00%	
0.05%	Dead	3	2	23	18	14	25	13	19	29	14	25	15
	Total	5	4	33	26	24	30	22	22	33	18	26	15
Avg. % Dead:		55.00%		69.46%		70.83%		72.73%		82.83%		98.08%	
0.10%	Dead	6	6	7	5	6	13	23	21	22	21	14	37
	Total	6	8	20	8	11	14	28	29	25	23	14	37
Avg. % Dead:		87.50%		48.75%		73.70%		77.28%		89.65%		100.00%	
Control	Dead	2	1	0	1	1	0	0	0	0	0	0	2
	Total	7	13	6	14	11	6	22	20	12	22	10	13
Avg. % Dead:		18.13%		3.57%		4.55%		0.00%		0.00%		7.69%	

Table 2: Trypan Blue exclusion assay results. Q1 and Q2 are separate quadrants on hemocytometer.

		30 Mins	1 Hr	2 Hrs	4 Hrs	8 Hrs	24 Hrs
0.01%	Well 1	109	345	501	498	526	1012
	Well 2	118	356	539	746	604	1007
Avg. MCP-1 Conc.:		113.5	350.5	520.0	622.0	565.0	1009.5
0.05%	Well 1	129	372	694	689	672	998
	Well 2	105	389	500	739	886	1016
Avg. MCP-1 Conc.:		117.0	380.5	597.0	714.0	779.0	1007.0
0.10%	Well 1	124	402	728	786	804	1014
	Well 2	121	392	872	863	1002	1098
Avg. MCP-1 Conc.:		122.5	397.0	800.0	824.5	903.0	1056.0
Control	Well 1	120	428	713	987	1084	992
	Well 2	61.8	71.4	84.3	98.7	84.7	98.3
Avg. MCP-1 Conc.:		90.9	249.7	398.7	542.9	584.4	545.2

Figure 3: MCP-1 production after exposure to 1-octen-3-ol (pg/mL).

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